

The University of Chicago

VITAMIN C AND RESISTANCE OF THE
GUINEA PIG TO INFECTION WITH
BACTERIUM NECROPHORUM

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY

1937

By
NORMAN B. McCULLOUGH

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Reprinted from
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July-August, 1938, Vol. 63, pp. 34-53

GEORGE BANTA PUBLISHING Co., MENASHA, WIS.

VITAMIN C AND RESISTANCE OF THE GUINEA PIG TO INFECTION WITH BACTERIUM NECROPHORUM

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INTRODUCTION

The concept that diet may be intimately concerned with resistance to bacterial infections has been entertained by clinicians for some time. It is only within the last twenty years, however, that experimental work has appeared in substantiation of these claims. The recent interest in the subject is a direct outgrowth of the research conducted during this period in the field of nutrition, and in particular, of the discovery of the so-called vitamins and their relations to deficiency diseases. A large mass of literature has accumulated dealing with both experimental and clinical observations. Much of this work is very difficult to evaluate.

The literature concerning the influence of vitamin C upon resistance to infection is somewhat unsatisfactory and contradictory. The numbers of animals used by many investigators have been too small to lend significance to the results. In many instances, the diets used in animal experimentation may be criticized either from the standpoint of not being deficient in vitamin C or of failing to provide adequately in all other respects for the nutritional requirements of the animal. The use of crude sources of the vitamin, such as orange or lemon juice, rendered necessary by the nonavailability of the pure vitamin, adds confusion to the earlier work and perhaps explains some of the discrepancies and contradictions noted, since these sources may well contain substances other than vitamin C which may affect resistance. It has been impossible to gauge accurately the dose of vitamin administered. These criticisms have been largely eliminated from the recent investigations by the isolation, identification, and synthesis of the vitamin and its ready availability as a pure crystalline chemical entity.

In much of the work virulent organisms, such as the tubercle bacillus, have been employed and the survival time of the animals recorded. Since either process in itself—that is, the infection or the vitamin deficiency—terminates fatally and the survival time of the animal is subject to extreme variation, this method does not give a satisfactory index of the effect upon resistance of a substance as intimately concerned with the general cellular metabolism as is vitamin C.

HISTORICAL REVIEW

As far back as 1912, Nicolle¹ remarked upon the prevalence of spontaneous pneumonia in guinea pigs fed on poor diets. Theobald Smith² also noted

Received for publication, February 25, 1938.

1. Thèse de Paris, no. 78. 1912.

2. J. M. Research 29: 291, 1913.

a high incidence of epizootic pneumonia in his colonies of guinea pigs at certain times of the year. He observed that these epizootics promptly disappeared when fresh green fodder was added to the diet. These men did not comment as to the specific dietary factor involved nor did they report scurvy as being present in the animals. Later workers have attributed these findings, which have been well substantiated, to latent scurvy in the animals.

The first experimental work was done by Jackson and Moody,³ who recovered a green producing streptococcus from the organs of scorbutic guinea pigs. When injected into normal guinea pigs and rabbits, this organism gave rise to lesions in the bones, lymph glands, and gums. They were able to recover it 40 days after inoculation. In the scorbutic guinea pig, this organism caused a marked progression of the scorbutic lesions and death of the animal. Upon studying the effect of chronic scurvy upon resistance to infection, Findlay⁴ found scorbutic guinea pigs to be susceptible to a smaller inoculum of the organisms used than were the normal animals. He administered the pneumococcus, *Staphylococcus aureus*, *Streptococcus hemolyticus*, and *Bacterium coli* intraperitoneally. He stated that this lowering of resistance was not acute but was consistent throughout the experimental series. He postulated that this decrease in resistance may be in part due to the degenerative changes and feeble leukoblastic reaction found in the bone marrow of chronically scorbutic guinea pigs. Werkman, Nelson and Fulmer,⁵ using the pneumococcus and *Bacillus anthracis*, also found the scorbutic guinea pig to be slightly less resistant than the normal. They stated that a determinable though not a marked break in resistance occurs; that a lowering of the body temperature (rectal) in the scorbutic guinea pig is correlated with and is of primary significance in explaining it. Wámoscher⁶ studied the occurrence of spontaneous pneumonia in a group of 400 guinea pigs, following the course of infection with roentgen ray. His experiments indicated that chronic (subacute) scurvy in the guinea pig is a predisposing factor in spontaneous pneumonia. Many of the organisms isolated were not pathogenic for the normal animal. Administration of large quantities of orange juice resulted in cure in some instances. Schmidt-Weyland and Költzsch⁷ attempted to induce infection in scorbutic guinea pigs by droplet inhalation of pneumococci and *Bacterium suipestifer*. Their results are confusing since the animals normally harbor these organisms. The incidence of infection was practically the same whether the scorbutic animal was artificially inoculated or not. However, infection did not occur until a rather severe scorbutus developed.

By far the greatest amount of experimental work has been done on tuberculosis. Mouriquand and coworkers⁸ were unable to demonstrate any influence of scorbutus on tuberculosis in the guinea pig, or vice versa. On the other hand, Mouriquand, Roचाix, and Dosdat⁹ found that when large doses of

3. J. Infect. Dis. **19**: 511, 1916.

4. J. Path. and Bact. **26**: 1, 1923.

5. J. Infect. Dis. **34**: 447, 1924.

6. Ztschr. f. Hyg. u. Infektionskr. **107**: 240, 1927.

7. Ztschr. f. Hyg. u. Infektionskr. **108**: 199, 1927.

8. Compt. rend. Soc. de biol. **87**: 854, 1922; **88**: 1043, 1923; **91**: 205, 1924.

9. Compt. rend. Soc. de biol. **93**: 901, 1925.

virulent organisms were employed, the infection evolved more rapidly than in the controls. Coulard¹⁰ reported that vitamin C deficient guinea pigs, inoculated with small doses of tubercle bacilli, developed a more severe infection than normals, with more rapid involvement of the viscera. Using guinea pigs only partly deficient in vitamin C, Bieling,¹¹ Heymann,¹² and Basu¹³ showed that a prescorbutic state shortened the lives of animals having a progressive tuberculous infection. The chronically deficient guinea pigs died considerably earlier than the tuberculous controls. According to Bieling,¹⁴ guinea pigs with chronic tuberculosis are more easily precipitated into acute scurvy. McConkey and Smith,¹⁵ studying intestinal tuberculosis in the guinea pig fed tubercular sputum, presented evidence that a diet partially deficient in vitamin C renders the animal much more susceptible to the development of ulcerative intestinal tuberculosis. Greene and coworkers,¹⁶ concluded from their work that "chronic vitamin C deficiency combined with a progressive tuberculous infection causes a significant shortening of the survival period and a significant decrease in the body weight of guinea pigs." Likewise, Scoz and Cattaneo¹⁷ found a lowering of resistance to experimental tuberculosis in the vitamin C deficient guinea pig.

The effect of hypervitaminosis C in animal tuberculosis has been studied by several investigators. Leichtentritt¹⁸ observed that large amounts of orange juice increased the survival time of tuberculous guinea pigs. His treated animals lived twice as long as the tuberculous controls on a normal diet. Grant¹⁹ reported a decrease in the extent and severity of pulmonary lesions in tuberculous guinea pigs when the amount of vitamin C in the diet was increased. Bella²⁰ failed to find that a superior degree of protection was afforded tubercular guinea pigs given orange juice or small amounts of ascorbic acid (crystalline vitamin C) in addition to a normal diet. The observations of Heise and Martin²¹ did not disclose any alteration in the course of experimental tuberculosis in the guinea pig when a large excess of vitamin C was injected intravenously for a period of several months.

Another problem which has been investigated intensely of late is the relation of scurvy to rheumatic fever and rheumatoid arthritis. Rinehart and Mettier²² and Rinehart, Conner, and Mettier²³ produced lesions in the heart valves and joints of guinea pigs by a combined scurvy and superimposed in-

10. *Presse méd.*, **31**: 611, 1925.

11. *Ztschr. f. Hyg. u. Infektionskr.* **101**: 442, 1924.

12. *Klin. Wchnschr.* **5**: 59, 1926.

13. *Ztschr. f. Vitaminforsch.* **3**: 91, 1934.

14. *Deutsche med. Wchnschr.* **53**: 182, 1927.

15. *J. Exper. Med.* **58**: 503, 1933.

16. *Am. J. Dis. Child.* **50**: 281, 1935; *Am. Rev. Tuberc.* **33**: 585, 1936

17. *Boll. d. Soc. ital. di biol. sper.* **11**: 909, 1936.

18. *Ztschr. f. Hyg. u. Infektionskr.* **102**: 388, 1924.

19. *Am. Rev. Tuberc.* **21**: 115, 1930.

20. *Boll. d. Soc. ital. di biol. sper.* **10**: 141, 1935.

21. *Proc. Soc. Exper. Biol. & Med.* **35**: 337, 1936.

22. *Am. J. Path.* **9**: 932 and 952, 1933; **10**: 61, 1934.

23. *J. Exper. Med.* **59**: 97, 1934.

fection with beta type streptococci, which they describe as similar to those found in rheumatic fever and rheumatoid arthritis in man. In the heart valves they obtained combined degenerative and proliferative lesions resembling those of acute rheumatic fever. Neither the scurvy nor the infectious agent alone produced comparable lesions. This work has been continued by Rinehart,²⁴ using other strains of hemolytic streptococci, gamma streptococci, and *Bacterium bronchisepticus*, with similar findings. Clinical investigations by Rinehart and coworkers²⁵ and by many others have substantiated the postulation that a vitamin C deficiency exists in such pathological conditions in man. The implications of this work are so far-reaching that they have stimulated considerable research on the part of other investigators. Many clinical observations pro and con have been made on the subject; but the majority of observers agree that a vitamin C deficiency accompanies rheumatic fever and similar conditions in a large proportion of the cases. It is interesting to note in this connection the work of Daniels and Everson.²⁶ When they administered acetylsalicylic acid to young children, the urinary excretion of vitamin C was increased. Discussing the work of Rinehart and associates, they suggest that the occurrence of scorbutic symptoms in such cases may be due to the use of salicylates in treatment.

Stimson, Hedley, and Rose²⁷ described lesions in scorbutic guinea pigs, produced by the injection of streptococcic toxin, which they claim closely resemble lesions found in some stages of human rheumatic heart disease. Schultz,²⁸ using beta streptococci in the scorbutic guinea pig, produced a nonpurulent carditis and a valvulitis, accompanied by proliferative changes. These lesions were not seen if the animal had developed an immunity towards streptococcal infections before the onset of scurvy. He described the lesions as only slightly resembling those seen in rheumatic fever. According to McBroom, Sunderland, Mote, and Jones,²⁹ degenerative and proliferative valvular changes occur in the heart in the majority of guinea pigs dying from scurvy, whether or not there is superimposed infection. They do not consider there is any evidence relating heart changes in guinea pig scurvy to lesions found in rheumatic fever in man. They state that cellular infiltration and subendothelial proliferative reactions are common to both rheumatic fever and scurvy. Mouriquand and others³⁰ report that guinea pigs, receiving diets partially deficient in vitamin C for one hundred days and longer, developed an ankylosing rheumatic syndrome in addition to the usual symptoms of scurvy. This condition was only slightly or not at all influenced by large doses of vitamin C.

There are scattered observations on some few other infections. Zinsser, Castaneda, and Seastone³¹ found that the vitamin C deficient guinea pig de-

24. *Ann. Int. Med.* **9**: 586 and 671, 1935; *J. Lab. & Clin. Med.* **21**: 597, 1936.

25. *Proc. Soc. Exper. Biol. & Med.* **35**: 347 and 350, 1936.

26. *Proc. Soc. Exper. Biol. & Med.* **35**: 20, 1936.

27. *Pub. Health Rep.* **49**: 361, 1934.

28. *Arch. Path.* **21**: 472, 1936.

29. *Arch. Path.* **23**: 20, 1937.

30. *Compt. rend. Acad. d. sc.* **204**: 921, 1937.

31. *J. Exper. Med.* **53**: 333, 1931.

veloped a more severe infection with a Mexican strain of typhus than did normal controls. The *Rickettsiae* in exudates were far more numerous than usual and many were extracellular. Giroud³² observed that vitamin C deficient guinea pigs, infected with typhus virus, showed a disturbance in the cyclical course of the infection. The deficient animals developed the infection more rapidly, symptoms appearing the second or third day after inoculation; while in the normal animal, the onset was not until the seventh or eighth day. A large number of the animals developed orchitis, which is rare in the guinea pig on a vitamin C adequate diet. Takahashi³³ reported that young rabbits and guinea pigs on a vitamin C deficient diet were more susceptible to infections with staphylococci and tended to develop abscesses at the ends of the long bones. Introduction of foreign substances into the circulation resulted in retention in the metaphysis of the long bones. He suggested that this stagnation accompanying vitamin C deficiency may be a mechanism explaining the occurrence of acute osteomyelitis. Jungeblut³⁴ reported that crystalline vitamin C neutralized the virus of poliomyelitis in vitro. In subsequent work³⁵ attempting to treat poliomyelitic monkeys with vitamin C, he found that the vitamin in proper doses possessed distinct therapeutic properties in experimental poliomyelitis, but was without effect as a prophylactic. It is his belief that vitamin C deficiency may play a part in susceptibility to the virus of poliomyelitis. Almon, Pessin, and Stovall³⁶ found no increase in susceptibility of guinea pigs to *Monilia albicans* on a vitamin C deficient diet. Perla³⁷ has studied the effect of repeated injections of cevitamic acid (vitamin C) in guinea pigs infected with *Trypanosoma brucei*, and concluded that such treatment raised the resistance of the animals to the infecting agent.

Many clinical papers on vitamin C metabolism in infectious diseases have appeared. Since many of the reports are controversial and involve too few observations to be statistically significant, it seems of little value to review them at length. The majority of observers agree that febrile diseases are accompanied by a depletion of the vitamin C reserves of the body. This is evidenced by a reduced urinary excretion of the vitamin, a reduced blood plasma value, and a reduced amount excreted in the urine following administration of large amounts of the crystalline substance. The clinical reports have been made on such conditions as pneumonia, tuberculosis, inoculation malaria, rheumatic syndromes, and a few other infections. The sum total of these observations constitutes a mass of data which is of sufficient magnitude and general agreement to allow us safely to conclude that the febrile conditions such as those noted above do require a greater amount of vitamin C to maintain the nutritional balance than is required in the normal animal. Considering that vitamin C is intimately concerned with cellular respiration, it is not

32. Compt. rend. Soc. de biol. **121**: 714, 1936.

33. Arch. f. klin. Chir. **181**: 103, 1934.

34. J. Exper. Med. **62**: 517, 1935.

35. J. Bact. **31**: 34, 1936; J. Exper. Med. **65**: 127, 1937.

36. J. Infect. Dis. **59**: 54, 1936.

37. Unpublished. Referred to in review by Perla, Arch. Path. **23**: 543 and 683, 1937.

surprising that this is so. Any disturbance or increase in cellular metabolism, such as in a febrile condition, would have an effect on the utilization of the vitamin. Clinical therapy, based on supplying this need, has not been markedly successful.

Having investigated the experimental work relating vitamin C deficiency to resistance to infection, and having found it extremely suggestive though not definitely conclusive, it will be interesting to consider the work bearing on the theoretical aspects of the lowering of resistance; that is, the effect on the recognized defensive mechanisms of the body.

Harde³⁸ was the first to show that vitamin C possesses antitoxic properties. She obtained a marked degree of protection to diphtheritic intoxication in the guinea pig by administration of crystalline vitamin C. Later, Harde and Philippe³⁹ found that vitamin C inactivates diphtheria toxin in vitro as well as in vivo. Both in vitro and in vivo neutralization of diphtheria toxin by vitamin C were further confirmed by Greenwald and Harde,⁴⁰ and Jungeblut and Zwemer,⁴¹ using stable standardized toxins. Since the appearance of these early reports, a score of other workers have verified this antitoxic property of vitamin C.

Hochwald⁴² has shown that vitamin C, administered previously to or concurrently with the injection of the shocking dose into the pericardium of sensitized guinea pigs, completely protected against or greatly lessened the anaphylactic reaction to the reinjection of the antigen. This antianaphylactic property of vitamin C has been confirmed by several workers.⁴³ Giroud and Giroud,⁴⁴ have shown further that injection of ascorbic acid into the sensitized rabbit (an animal not susceptible to scorbutus) previous to reinjection of the antigen, also gives protection against anaphylactic shock. Hochwald,⁴⁵ has demonstrated that this same protective effect is produced by other substances: cystine, glutathione, and sodium thiosulphate. He ascribes it to the redox potential of these substances.

That skin reactions to tuberculin in scorbutic tuberculous guinea pigs were definitely diminished, as compared to normally fed tuberculous animals, was reported by Bieling.⁴⁶ Prausnitz and Schilf⁴⁷ confirmed this lessened skin reactivity.

Mouriquand, Rochaix, and Michel⁴⁸ were unable to prove the scorbutic guinea pig more susceptible to tuberculin shock than the normal. Steinbach and Klein⁴⁹ found that vitamin C failed to neutralize tuberculin in vitro, and

38. *Compt. rend. Acad. d. sc.* **199**: 618, 1934.

39. *Compt. rend. Acad. d. sc.* **199**: 738, 1934.

40. *Proc. Soc. Exper. Biol. & Med.* **32**: 1157, 1935.

41. *Proc. Soc. Exper. Biol. & Med.* **32**: 1229, 1935.

42. *Ztschr. f. d. ges. exper. Med.* **97**: 433, 1935; *Zentralbl. f. inn. Med.* **56**: 769, 1935.

43. *J. Immunol.* **31**: 209, 1936; *Compt. rend. Soc. de biol.* **123**: 1146, 1936; *Monatschr. f. Kinderh.* **67**: 244, 1936.

44. *Compt. rend. Soc. de biol.* **121**: 1588, 1936; *Compt. rend. Soc. de biol.* **122**: 537, 1936.

45. *Ztschr. f. d. ges. exper. Med.* **98**: 578, 1936; *Klin. Wchnschr.* **15**: 894, 1936.

46. *Ztschr. f. Hyg. u. Infektionskr.* **104**: 518, 1925.

47. *Deutsche med. Wchnschr.* **50**: 102, 1924.

48. *Compt. rend. Soc. de biol.* **91**: 208, 1924.

49. *Proc. Soc. Exper. Biol. & Med.* **35**: 151, 1936.

that vitamin C administered to tuberculous animals failed to reduce the skin reactivity to tuberculin. However, they did observe an increased tolerance to small amounts of tuberculin given over a prolonged period to tuberculous guinea pigs treated daily with injections of vitamin C.

Zolog⁵⁰ reported that vitamin C deficient guinea pigs, sensitized to horse serum, were less sensitive to reinjection of the antigen than were the control animals. Hurwitz and Nicholls,⁵¹ and Hurwitz and Wessels,⁵² in studying the reactivity of smooth muscle in guinea pigs sensitized to horse serum, discovered that uteri of guinea pigs on a vitamin C deficient diet were much less reactive to the homologous antigen than were the controls. These uteri also failed to respond to histamine, pituitary extract, and barium chloride, whereas the control uteri reacted with all these substances. No difference appeared in the reactivity of bronchial smooth muscle.

Several workers have postulated a relationship between vitamin C and serum complement. The opinions on this subject are by no means in accord. Zilva,⁵³ and Hamburger and Goldschmidt⁵⁴ could detect no difference in the complement content of scorbutic and normal guinea pigs. Koch and Smith⁵⁵ observed an increase in complement in scorbutic animals. Simola,⁵⁶ Simola and Brunius,⁵⁷ and Chakraborty⁵⁸ report no change in complement in the scorbutic guinea pig. On the other hand, Harde and Thomson⁵⁹ found that guinea pigs given diphtheria toxin, which should reduce the vitamin C content, showed a reduction in complement. Also, when a large dose of vitamin C was injected intravenously into a rabbit one hour before bleeding, the serum was much more active than the normal. The number of animals used in this work was too small to lend significance to the results. Marsh⁶⁰ states that, in guinea pigs fed on a vitamin C deficient diet for 7 days, the complement undergoes reduction or entirely disappears. Horgan⁶¹ substantiates this conclusion. These workers believe vitamin C to be a precursor of serum complement.

Zilva⁵³ did not discover any impairment of the ability of scorbutic guinea pigs to form agglutinins against *Bacterium typhosum*. These results were confirmed by Werkman, Nelson, and Fulmer.⁵ They also investigated the phagocytic ability of the leukocytes from scorbutic guinea pigs against *Bacterium typhosum* and *Staphylococcus albus*. No difference between these animals and the controls was observed. Recently, Jusatz⁶² injected vitamin C

50. *Compt. rend. Soc. de biol.* **91**: 215, 1924.

51. *Proc. Soc. Exper. Biol. & Med.* **28**: 139, 1930.

52. *Proc. Soc. Exper. Biol. & Med.* **29**: 122, 1931.

53. *Biochem. J.* **13**: 172, 1919.

54. *Jahrb. f. Kinderh.* **100**: 210, 1923.

55. *Proc. Soc. Exper. Biol. & Med.* **21**: 366, 1924.

56. *Acta. Soc. med. fenn. duodecim.* **16**: 12, 1932.

57. *Biochem. Ztschr.* **258**: 228, 1933.

58. *Indian M. Gaz.* **72**: 23, 1937.

59. *Compt. rend. Acad. d. sc.* **200**: 1425, 1935.

60. *Nature* **137**: 618, 1936.

61. *Nature* **137**: 872, 1936.

62. *Klin. Wchnschr.* **14**: 1419, 1935.

concurrently with horse serum into rabbits, obtaining precipitins in much higher titer than when horse serum alone was injected.

Lawryncowicz⁶³ claims that in the scorbutic guinea pig the leukocyte production is reduced; aleuronat injected into the peritoneal cavity fails to call forth a leukocytic exudate. He also found a diminished phagocytic power of the leukocytes for *Bacterium coli* and the tubercle bacillus. More recently, that vitamin C deficiency in the guinea pig is accompanied by a marked leukopenia has been reported by Euler and Malmberg.⁶⁴

Grant⁶⁵ observed that the intestinal wall becomes more permeable to *Bacterium aertrycke* when the vitamin C to D ratio is extremely altered.

Smith and McConkey⁶⁶ reported an increased incidence of peptic ulcer in the vitamin C deficient guinea pig. They attributed this to failure of the mucosa to heal rapidly after mechanical injury.

In studying severe operative head wounds in the scorbutic guinea pig, Jeney and Korpásky⁶⁷ discovered no disturbance of new skin formation, but the rebuilding of connective tissue and bone was seriously disturbed and retarded. Similarly, bone regeneration in the scorbutic guinea pig was, according to Hanke,⁶⁸ greatly retarded.

Administration of vitamin C to rabbits and guinea pigs after bone resection accelerated bone regeneration. Baker⁶⁹ found that the addition of vitamin C to tissue cultures of chicken monocytes stimulated proliferation. She concluded that vitamin C is necessary for the proliferation of monocytes in vitro. Similarly, Jeney and Törö⁷⁰ observed that vitamin C stimulated the production of fibers by fibroblasts in tissue culture. They considered that this is evidence that vitamin C is necessary for the production of intercellular cement and connecting fibers. It will be recalled in this connection that Wolbach and Howe⁷¹ characterized scorbutus as the inability of supporting structures to produce and maintain intercellular substance.

Tschassovnikow and Zelikovskaja⁷² report that vitamin C deficiency delayed the absorption and elimination of trypan blue, and postulate that this retardation is due to the effect of vitamin C deficiency on the reticulo-endothelial system. In this connection, Locke, Locke, Bragdon, and Mellon⁷³ found that vitamin C administered intravenously to the rabbit previous to injection of pneumococci, resulted in more rapid elimination of the organisms from the blood stream.

EXPERIMENTAL METHOD

Various strains of nonsporulating anaerobes were used in this study. These organisms are

63. J. de physiol. et de path. gén. **29**: 270, 1931.

64. Ztschr. f. physiol. chem. **243**: 121, 1936.

65. J. Infect. Dis. **39**: 502, 1926.

66. Arch. Int. Med. **51**: 413, 1933.

67. Zentralbl. f. chir. **61**: 2836, 1934.

68. Deutsche Ztschr. f. chir. **245**: 530, 1935.

69. Compt. rend. Soc. de biol. **121**: 427, 1936.

70. Virchow's Arch. f. path. Anat. **298**: 87, 1936.

71. Arch. Path. **1**: 1, 1926.

72. Méd. exp. Ukraine **12**: 16, 1935.

73. Science **86**: 228, 1937.

sometimes found in nature associated with chronic necrotic processes in man and other animals. Dack and associates⁷⁴ have studied this group of organisms in relation to chronic ulcerative colitis in man. Their role as etiologic agents in this disease is not clear, but these investigators have been able to isolate such organisms from lesions in the colon in most cases of chronic ulcerative colitis. During the eruptive stages of the disease, they are sometimes present in pure culture. There seems to be little agreement as to nomenclature of these organisms. As stated by Dack and others,⁷⁵ workers in veterinary bacteriology generally have used the name *Bacterium necrophorum*, while those studying disease in man have preferred the name *Bacterium funduliforme*. In a complete study of strains of this group of organisms isolated from both human and animal sources, Dack and others⁷⁶ have shown that it is impossible to differentiate species within this group by methods commonly in use for bacterial classification. Reasons have been given for retaining the name *Bacterium necrophorum* which has been used by Dack in all previous work with organisms of this group.

The strains of *Bacterium necrophorum* used in the present investigation were obtained from the following sources: strains 101, 103, 104, 107, and 110 from human cases of chronic ulcerative colitis; strains 116 from a pig, 117 from a sheep, 118 and 119 from *Macaca mulatta* monkeys and 121 from a *Sphinx* baboon (all these animal strains were obtained from experimental traumatic lesions in the colons of the respective animals); strains 123 to 134 from liver abscesses of cattle that had been slaughtered. These strains graded in virulence for rabbits from those that were nonpathogenic (or ones producing only transient abscesses in the case of the human strains), to strains that were lethal for the rabbit (bovine strains). A complete study of these strains has been made by Dack and associates.⁷⁵

Young guinea pigs weighing approximately 250 gm. at the start of each experiment were used throughout the experimental series. These animals were placed on a vitamin C deficient stock diet of the following composition:

Whole milk powder.....	40.5%
(Inactivated at 120 C. for two hours)	
Ground rolled oats.....	25.0%
Bran.....	25.0%
Dried brewers' yeast (Fleischmann).....	5.0%
Cod liver oil (Mead, plain standardized).....	3.0%
Sodium chloride.....	1.0%
Ferric citrate.....	0.5%

In addition, the control animals were given two milligrams per day of crystalline vitamin C (Cebione, Merck) orally. A fresh stock solution of the vitamin in 5% acetic acid was prepared each week and kept at refrigerator temperature. This stock was diluted as needed to yield a solution containing 4 mg. per cc. of vitamin C. The control animals were given 0.5 cc. of this solution daily with a pipette. The animals were weighed 3 times per week.

Preliminary trial revealed that animals on the deficient diet developed the usual signs of acute scurvy in about fourteen days, with death occurring in from three to five weeks. Figure 1 shows the average weight changes for both the control and the deficient animals under these conditions. As these data are typical of the weight records for the entire series of experiments, detailed weight records will be omitted.

In a preliminary experiment, guinea pigs in groups of 10 each were placed on the deficient diet for various periods of time (7, 14, and 21 days), their weight records kept, and at the end of the prescribed periods vitamin C was replaced in their diet. Since it has been shown that scorbutic animals require rather large amounts of the vitamin to recover from the scorbutic condition, the animals were given 5 mg. of vitamin C daily. Those having been on the deficient diet for seven days rapidly returned to normal and gained weight. The group on the deficient diet for 14 days made a slower recovery, with 2 fatalities. These recovered animals showed the results of the scorbutic condition in the form of stiffness in the knee and elbow joints. In the case of the third group which had been on the deficient diet for 3 weeks and were already in the

74. Arch. Surg. **31**: 225, 1935; J.A.M.A. **106**: 7, 1936; J. Infect. Dis. **60**: 335, 1937.

75. To be published.

final stages of acute scurvy and extremely moribund, only 1 animal lived. The rest succumbed to scurvy within 2 to 14 days following treatment, in spite of the vitamin therapy. In consequence of this, it was felt that guinea pigs on a vitamin C deficient diet for longer than one week prior to inoculation were useless as test animals, since the acute scurvy itself would limit the length of survival and mask any effects of the infectious agent. Therefore the technic adopted and retained throughout the series consisted of placing the guinea pigs on the deficient diet and making all inoculations on the seventh day thereafter. All inoculations were made subcutaneously on the shaved ventral surface of the animals.

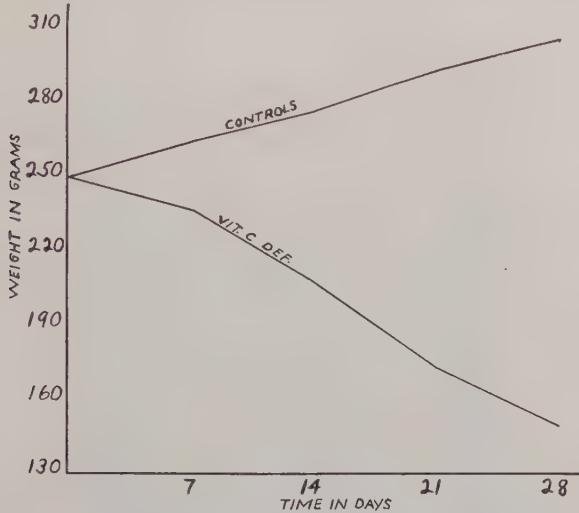


Fig. 1.—Showing average weights of vitamin C deficient guinea pigs, and of control guinea pigs receiving 2 mg. per day of crystalline vitamin C.

Twenty-four hour cultures of the organisms grown in Rosenow's dextrose brain broth medium were used. The supernatant fluid containing the organisms was employed for inoculation. Such a culture will be referred to in the following pages simply as a "culture" of the organisms. When large numbers of organisms were desired, they were removed from this medium by centrifugation and resuspended in a small amount of the freshly boiled sterile Rosenow's broth. This technic was standardized as accurately as possible. Estimates of the numbers of organisms were made by dilution, using Rosenow's medium. All such suspensions used in the investigation contained at least one billion viable organisms per cc. Subsequently, such a suspension will be known as a "concentrated suspension" of the organisms in question.

For purposes of culturing these organisms from lesions produced in the guinea pigs, 2 methods were used:

(1) Material from the lesions was streaked on 10% sheep blood veal infusion agar plates. These plates were incubated in an anaerobic jar containing an alkaline solution of pyrogallol. The oxygen was exhausted from the jar, replaced with carbon dioxide, which in turn was exhausted, leaving a 5% carbon dioxide residuum.

(2) Small pieces of tissue from the lesions were removed aseptically and dropped into tubes of freshly boiled and cooled Rosenow's dextrose brain broth medium. All cultures were incubated at 37 C.

Tissue sections were taken at necropsy immediately after the animals were sacrificed. They were fixed in Bouin's picroformol solution, dehydrated and imbedded in the usual manner, sectioned, and stained with eosin-hematoxylin.

EXPERIMENTS WITH HUMAN STRAINS OF BACTERIUM NECROPHORUM

In this experiment 4 different strains were used: 103, 104, 107, and 110. Five vitamin C deficient guinea pigs each were injected subcutaneously with

1.0 cc. of a culture of the respective strains. An equal number of the control animals receiving crystalline vitamin C in addition to the stock diet were similarly injected.

The majority of the vitamin C deficient animals developed lesions at the site of injection, the lesion first appearing in twenty-four hours as a small, firm lump beneath the skin. This area remained firm and the lesion spread in all directions from this initial point. From the exterior it appeared as a darkened (bluish-red) area beneath the skin and firmly adherent to it (fig. 2).



Fig. 2.—Vitamin C deficient guinea pig 10 days after subcutaneous inoculation with strain 110.

This lesion in all cases persisted and continued to spread until the termination of the experiment. The control guinea pigs developed a slight inflammatory reaction at the site of injection, which appeared in 24 hours, persisted for 1 or 2 days, and entirely disappeared. Two weeks after inoculation, all the animals were still alive in both the experimental and the control series. Owing to the acute scorbutic condition of the vitamin C deficient guinea pigs, the experiment was terminated at this point, the animals killed and autopsied.

In the deficient series, all animals inoculated with strains 103 and 110, and one each from the groups receiving strains 104 and 107, had the above described lesion in varying degrees. The area involved varied from one measuring 2 by 2 cms. to one measuring 6 by 13 cms., extending over nearly the whole surface of the abdomen. At autopsy, this lesion appeared as a dark red, firm, fibrous growth between the skin and the muscle wall, measuring from 2 to 5 mm. in thickness and adhering firmly to the skin. Microscopic

examination of sections showed the external darkening to be due to the high vascularity of the tissue. Two guinea pigs infected with strain 103 developed abscesses containing pus.

All lesions were cultured both aerobically and anaerobically on blood agar plates and by dropping aseptically handled portions of the tissue into Rosenow's medium. From those lesions containing pus, *Bacterium necrophorum* was isolated in pure culture on the anaerobic blood agar plates. All the other cultures remained sterile.

Since the organisms in question are very sensitive to atmospheric oxygen and require a heavy initial inoculum for growth to be obtained, it was felt that the failure to isolate the organisms, except in the 2 cases where pus was present, in no way precluded their presence in the lesions. Accordingly, 10 deficient guinea pigs were injected with 1.0 cc. of a culture of strain 103 (which seemed to be the most virulent) that had been inactivated by heating in a water bath at 60 C. for one hour. Five control animals were similarly injected. These control animals developed slight inflammatory zones at the site of injection, which appeared in 24 hours and completely disappeared in 2 or 3 days. The deficient animals behaved likewise, except that it was noted that the initial inflammatory response was delayed, usually not appearing until forty-eight hours after injection. A small hyperemic spot beneath the skin was, in most instances, discernible ten days later at autopsy. It thus appeared that inactivated cultures did not produce this proliferative lesion.

The effect of a large inoculum of organisms was studied. Concentrated suspensions of 5 strains—101, 103, 104, 107, 110—were used. Inoculations were made in the usual manner. Ten deficient and 10 control guinea pigs were inoculated with each strain, allowed to live for 14 days thereafter, sacrificed, examined, and the lesions cultured. The results were much the same as in the first experiment. In the vitamin C deficient guinea pig, despite the large number of organisms used for injection, the only difference observed was a larger number of typical abscesses containing pus (fig. 3). Measurements of the lesions varied from 2 by 2 cms. to 3 by 5 cms. All the guinea pigs receiving strain 103 had lesions; 6 of these contained pus. With strain 110, 8 guinea pigs had lesions with 4 containing pus. With strain 104, 9 guinea pigs had lesions with 5 containing pus. With strain 107, 5 guinea pigs had lesions with 2 containing pus. No specific lesions developed with strain 101.

Upon culturing these lesions, *Bacterium necrophorum* was recovered from those abscesses where pus was present. In one case (110), a staphylococcus was also obtained. In all the other instances, *Bacterium necrophorum* was present in pure culture. The heart blood from several of these guinea pigs was cultured with negative results. As previously, the control animals developed no infections whatever.

To summarize, of 50 vitamin C deficient guinea pigs injected with various (5) strains of *Bacterium necrophorum*, the specific organism was recovered in pure culture from the lesions arising in 16 of the animals, 15 others developed proliferative lesions from which the organism was not recovered, 3 died prematurely of nonspecific causes, while 15 remained uninfected. The 50 control animals failed to develop infections. It is emphasized that these

lesions in all cases were very low grade infectious processes, not causing a significant weight loss in the animals as compared to comparable uninfected vitamin C deficient guinea pigs. Even with a large inoculum of organisms, no very severe localized process was obtained and no generalization of infection occurred.

EFFECT OF SUBACUTE SCURVY ON INFECTION WITH
BACTERIUM NECROPHORUM

The effect of subacute scurvy in relation to infection in the guinea pig was studied. Forty-five guinea pigs were reduced to a condition of subacute



Fig. 3.—Vitamin C deficient guinea pig 14 days after subcutaneous inoculation with a concentrated suspension of strain 104. The typical discoloration evident in fig. 2 does not show well in this photograph.

scurvy by depriving them entirely of vitamin C for one week, to allow depletion of their vitamin C reserves. They were then given 0.25 mgms. of crystalline vitamin C daily during the rest of the experiment. These animals were definitely vitamin C deficient and but barely protected from the appearance of acute scurvy. A group of 5 uninfected guinea pigs so treated remained in this state without showing signs of acute scurvy but not gaining weight for the entire duration of the experiment. At the end of 14 days after being placed on the deficient diet (and seven days after the commencement of vitamin C administration in minimal doses), these animals weighed considerably less than normal controls. At this stage the test group was inoculated with strain 103, 1.0 cc. of a concentrated suspension of organisms being used.



Fig. 4.—Rabbit 3 days after subcutaneous inoculation with strain 134.

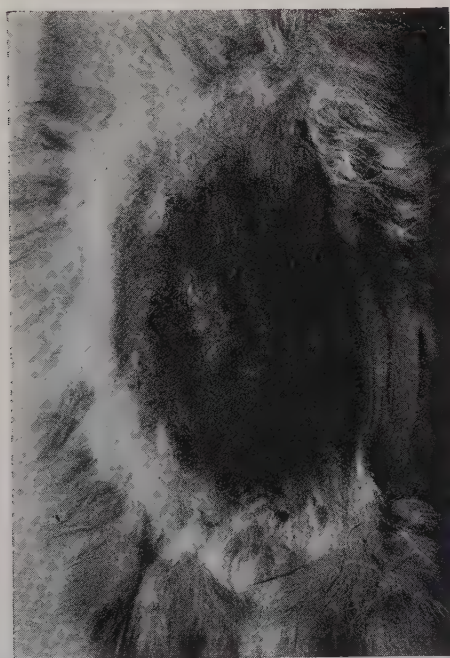


Fig. 5.—Rabbit 7 days after subcutaneous inoculation with strain 134.

As a control of the culture, 10 guinea pigs receiving the diet totally deficient in vitamin C (7 days) were similarly injected. The test animals were kept for a period of 3 weeks. It is significant that no infections developed during this period in the group receiving minimal amounts of vitamin C. The test animals were sacrificed and autopsied. The site of injection was barely discernible. Six of the ten animals on the totally deficient diet developed the typical lesion. Upon culturing these lesions, *Bacterium necrophorum* was recovered in three instances.

ORGANISMS CONTAINED IN PUS

Several human strains of *Bacterium necrophorum* were injected subcutaneously into rabbits, an animal relatively susceptible to infection with these organisms. In the rabbit, a large walled-off abscess containing a thick cheesy grey pus is produced. Of the several strains injected, one (104) yielded such an abscess. Ten days after inoculation the rabbit was anesthetized, the abscess opened aseptically, and the pus collected. Stained smears and cultures showed that the material contained large numbers of *Bacterium necrophorum* in pure culture. This pus was triturated in a sterile mortar with sterile cystine dextrose broth until a suspension that could be drawn into a syringe was obtained. Ten vitamin C deficient guinea pigs and an equal number of normal control animals were injected with this material. At the end of one week, all the deficient animals had the typical spreading abscesses. Pus appeared to be present in all cases. At this time, nine of the control animals had abscesses. These were more limited in extent than those in the deficient guinea pigs. Seven of these had ruptured to the surface and were draining. After 2 weeks, the controls had completely healed. The lesions in the deficient animals showed no tendency to heal. Only one abscess had ruptured to the surface. They were then sacrificed and the lesions cultured. Pure cultures of *Bacterium necrophorum* were recovered from 8 of the 10 animals.

STUDIES ON ANIMAL STRAINS

In this experiment the following animal strains were used: strains 116 (pig), 117 (sheep), 118 and 119 (monkeys), 121 (baboon), 123-134 (bovine liver strains). Two normal and 2 deficient guinea pigs each were injected with 1.0 cc. of the respective strains. Figs. 4 and 5 show the virulence of strain 134 on the rabbit.

The animal strains, with the exception of strain 117, produced abscesses in the vitamin C deficient guinea pigs in all instances. These lesions (fig. 6) developed, ruptured, and drained. All animals were alive at the end of 7 days. From the seventh to the tenth day the following animals died: those infected with strains 121, 124, 126, 127, 128, 129, 130, 132. These animals all had suppurating lesions showing no tendency to heal. The lesions measured 2 by 4 cms. to 6 by 6 cms. The remaining animals were sacrificed. The abscess of 1 guinea pig inoculated with strain 118 was fairly well healed. The other guinea pig receiving strain 118, and all the guinea pigs receiving strains 119, 123, 125, 131, and 133 had suppurative draining abscesses showing no tendency to heal. These animals were all weak and failing. Presumably they

would have died in a few days. Blood cultures were made from these sacrificed animals. These were all negative. There was no direct extension of lesions through the abdominal wall.

In the normal animals strains 116, 117, 127 failed to produce abscesses. One normal guinea pig infected with strain 124 did not develop an abscess. All the other control animals developed abscesses. These measured 1 by 1 cm. to 2 by 3 cm. One week later all these abscesses had ruptured to the surface and were healing. Ten days after inoculation the experiment was terminated. At this time complete healing had occurred in all the infected control animals



Fig. 6.—Vitamin C deficient guinea pig 7 days after subcutaneous inoculation with strain 134.

except in one instance (126) where a draining abscess still remained. The two guinea pigs infected with strain 128 died from undetermined causes before the completion of the experiment.

VITAMIN C THERAPY

Since the bovine strains were slightly pathogenic for the normal guinea pig, and infection could be expected in a higher percentage of the test animals, strain 134 was used for this experiment. Twenty vitamin C deficient guinea pigs were injected subcutaneously with 1.0 cc. of a culture of this strain. Pus-containing abscesses developed in all the animals. Seven days after inoculation these abscesses appeared as in fig. 5. At this stage vitamin C therapy was commenced. The animals were given 5.0 mg. of vitamin C per

day. One day later 2 guinea pigs died. They were autopsied and the lesions cultured. *Bacterium necrophorum* was present in pure culture. Three more guinea pigs died at the end of the fourth day. The abscesses in these animals had ruptured to the surface and were draining. The remaining animals recovered with complete disappearance of the lesions 10 days after the beginning of vitamin C therapy (fig. 7). The abscesses in 7 of these guinea pigs ruptured to the surface and the contents drained out. In the remaining animals this did not occur. All the guinea pigs were killed at this time and



Fig. 7.—Vitamin C deficient guinea pig, infected with strain 134, 10 days after the commencement of vitamin C therapy.

examined. Fibrous tissue had replaced the infected portion and cicatrization was occurring.

HISTOLOGICAL STUDY

For histological study a group of vitamin C deficient guinea pigs was infected with a strain (104) of *Bacterium necrophorum*. An animal was sacrificed each day thereafter and a section taken through the ensuing lesion. One week after infecting this group, the remaining animals were given 5 mgms. of vitamin C daily. Thus sections were obtained showing the development of the abscesses and their regression under vitamin C therapy. Control animals were similarly injected and sacrificed at daily intervals until those remaining showed no inflammatory reaction at the site of injection. Since no

abscesses developed in these control animals, sections were taken at the point of inoculation.

In the vitamin C deficient guinea pigs, histological examination revealed an initial infiltration of polymorphonuclear leukocytes. This cellular response was very slight in 24 hours, becoming more pronounced in those sections taken at the end of the second and third days. This stage was followed by a fibroblastic response at the periphery of the lesion, extending for some distance from the center which now contained a small amount of pus. After 7

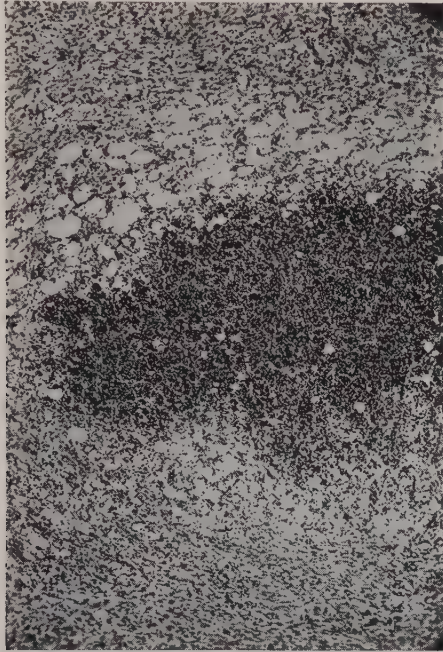


Fig. 8.—($\times 125$) Low power photomicrograph of abscess in a vitamin C deficient guinea pig 7 days after subcutaneous inoculation with strain 104.

days, this highly vascular granulation tissue spread for 2 to 3 cm. from the suppurative portion of the abscess (fig. 8). This walling off was not complete. There was no definite line of demarcation between the pus socket and the newly formed granulation tissue. Polymorphonuclear leukocytes were scattered sparsely throughout this new tissue. Perhaps this faulty walling off was due to the lack of production of fibers and intercellular cement substance in the scorbutic animal. Under vitamin C therapy these abscesses regressed and disappeared. Histologically there was disappearance of the pus, with a diminution in numbers and finally complete absence of polymorphonuclears from the area. The fibroblastic tissue changed in character, forming normal appearing granulation tissue. Ten days after the commencement of vitamin C therapy, there was only a thickening of the subcutaneous connective tissue layer (fig. 9).

In the control series, histological examination of the twenty-four hour

sections revealed a marked polymorphonuclear response as compared to that in the deficient guinea pig. The polymorphonuclears diminished steadily in numbers in the successive sections and were completely absent from those taken after the fourth day. A very slight fibroblastic proliferation occurred. Sections taken on the fourth and fifth days showed a slight production of fibroblasts, with a resulting thickening of the subcutaneous connective tissue layer. Since infection did not occur in these control animals, no guinea pigs were sacrificed after the fifth day.



Fig. 9.—($\times 125$) Low power photomicrograph of healed lesion (strain 104) 10 days after commencement of vitamin C therapy.

DISCUSSION

As pointed out in the historical review, the role of vitamin C in resistance to infection has been obscured by the technic of investigation. Most previous workers have used virulent organisms for experimental purposes. The experimental animal then would die from a summation of the injuries—the infectious process and the vitamin deficiency. As stated earlier, the author does not feel that such a technic gives results that can be satisfactorily evaluated. A scorbutic guinea pig is a very sensitive animal. Almost any injury in addition to the existing injury of the vitamin C deficiency will result fatally for the animal.

In the present investigation, strains of *Bacterium necrophorum* were used that were strictly nonpathogenic, as well as some strains that were slightly pathogenic for the normal guinea pig. With the human strains, no infections resulted in guinea pigs on a vitamin C adequate diet except in the one experiment where the organisms were contained in pus. Here, only minor

transient lesions developed, with rapid healing. In the scorbutic guinea pig, these human strains produced very minor abscesses. No noticeable weight change was observed that could be attributed to the effect of the infectious agent. There was no injury that threatened the life of the animal. If they were prevented from dying of acute scurvy by the timely replacement of vitamin C in the diet, all infected guinea pigs could be kept alive. Yet, with such a lowly virulent organism, extremely scorbutic guinea pigs became infected, whereas the control animals did not. These infections persisted until the animals died of scurvy or until vitamin C was replaced in the diet. This would appear to be good proof that the vitamin deficiency altered the resistance of the animals to infection with these human strains of *Bacterium necrophorum*. It is emphasized that throughout these experiments with the human strains, infection was produced only in extreme scorbutus. Even then the resulting processes, though persisting and spreading slowly, never endangered the lives of the animals, since they were doomed to die of acute scurvy long before these minor infections could possibly produce such injury. Guinea pigs having a subacute scurvy even bordering on acute scurvy failed to develop infection. Animals having well developed abscesses promptly recovered when vitamin C therapy was instituted.

When the slightly more virulent animal strains were used, more severe infections were produced in the deficient animals than in the controls. These lesions showed no tendency to heal as did those in the control animals. Many of these deficient guinea pigs died much sooner than did uninfected vitamin C deficient guinea pigs, although no generalization of infection occurred.

Many investigators have remarked upon the variability of organisms of this group as regards their pathogenicity for laboratory animals. So far as the guinea pig is concerned, perhaps some of this variability may be explained on the basis of the vitamin C balance of the animals used. Hallé⁷⁶ described spreading lesions in guinea pigs produced upon subcutaneous inoculation with an organism which he called *Bacillus funduliformis*. From his descriptions these lesions were very similar to those observed in the present instance in the vitamin C deficient guinea pig. It is impossible to know the nutritional state of his animals. It is suggested that perhaps vitamin C deficiency may have played a role in the production of these lesions.

SUMMARY

The relation of vitamin C deficiency in the guinea pig to infection with various strains of *Bacterium necrophorum* was studied. Avirulent human strains that did not produce infection in guinea pigs on a vitamin C adequate diet, produced minor abscesses in the extremely scorbutic guinea pig. Animals with a subacute scurvy did not develop infection. Vitamin C therapy resulted in prompt recovery of infected scorbutic animals. The slightly more virulent animal strains, which produced transient abscesses in the control animals, gave a more severe degree of infection in the vitamin C deficient guinea pigs. The lesions persisted and the guinea pigs died much sooner than did uninfected vitamin C deficient animals. A severe scorbutus was necessary before a drop in resistance to *Bacterium necrophorum* became evident.

76. Thèse de Paris, 1898.

